

## Improved Production of Heterologous Proteins by a Glucose Repression-Defective Mutant of *Kluyveromyces lactis*

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**The secreted production of heterologous proteins in *Kluyveromyces lactis* was studied. A glucoamylase (GAA) from the yeast *Arxula adeninivorans* was used as a reporter protein for the study of the secretion efficiencies of several wild-type and mutant strains of *K. lactis*. The expression of the reporter protein was placed under the control of the strong promoter of the glyceraldehyde-3-phosphate dehydrogenase of *Saccharomyces cerevisiae*. Among the laboratory strains tested, strain JA6 was the best producer of GAA. Since this strain is known to be highly sensitive to glucose repression and since this is an undesired trait for biomass-oriented applications, we examined heterologous protein production by using glucose repression-defective mutants isolated from this strain. One of them, a mutant carrying a *dgr151-1* mutation, showed a significantly improved capability of producing heterologous proteins such as GAA, human serum albumin, and human interleukin-1 $\beta$  compared to the parent strain. *dgr151-1* is an allele of *RAG5*, the gene encoding the only hexokinase present in *K. lactis* (a homologue of *S. cerevisiae* *HXK2*). The mutation in this strain was mapped to nucleotide position +527, resulting in a change from glycine to aspartic acid within the highly conserved kinase domain. Cells carrying the *dgr151-1* allele also showed a reduction in N- and O-glycosylation. Therefore, the *dgr151* strain may be a promising host for the production of heterologous proteins, especially when the hyperglycosylation of recombinant proteins must be avoided.**

Yeasts are very useful hosts for the production of heterologous proteins. The yeast *Kluyveromyces lactis* presents several advantages over other yeast species. It is positive for lactose fermentation, is able to grow on cheap substrates such as residual whey from dairy industries, and has competitive secretory properties, excellent large-scale fermentation characteristics, and food grade status; also, both episomal and integrative expression vectors are available for it (for reviews, see references 20, 40, and 50). Its ability to secrete heterologous proteins into the medium at a concentration higher than that secreted by *Saccharomyces cerevisiae* was demonstrated previously (50), although the secretory and glycosylation processes and their regulation are still poorly understood for *K. lactis* (1, 42, 43).

For *K. lactis*, the regulation of primary carbon metabolism differs markedly from that for *S. cerevisiae* and reflects the dominance of respiration over fermentation that is typical for the majority of yeast species (7). In *K. lactis*, respiration is not repressed by glucose, and fermentative and oxidative metabolism can take place simultaneously. Glucose repression, however, does exist: several enzymes that are required for alternate carbohydrate metabolism have been shown to be subject to glucose repression (6, 13, 17, 25, 30). The *K. lactis* genes in-

involved in glucose repression include *RAG1*, encoding a low-affinity glucose permease (23, 48); *DGR151* (or *RAG5*), encoding the single hexokinase of this yeast (34), and *KLMIG1*, encoding a component of the repressor complex acting on glucose-repressed genes (11).

The *rag1* and *dgr151-1* mutants are both non-glucose-repressible pleiotropic mutants (25, 47). They are also impaired in fermentative metabolism and require respiration for growth on glucose (22, 34, 49); the *Klmig1* $\Delta$  mutant is instead impaired in glucose repression only for the Lac/Gal pathway (14).

Notwithstanding the available knowledge on *K. lactis* physiology, no connections between the secretion and glycosylation pathways and between energy and carbon metabolism in this yeast have been explored.

We have therefore analyzed the secretory capabilities of *K. lactis* mutants affected in the genes mentioned above. Among these mutants, a notable phenotype was observed for the *dgr151-1* (*rag5*) mutant: this mutation resulted in a slight defect in glycosylation and a significantly improved capability in the secreted production of heterologous proteins.

### MATERIALS AND METHODS

**Strains and growth conditions.** The yeast strains used and their relevant genotypes are listed in Table 1. YP medium contained 5 g of Difco yeast extract and 10 g of Difco Bacto Peptone per liter. Minimal medium contained 0.7 g of yeast nitrogen base without amino acids (Difco) liter<sup>-1</sup> supplemented with appropriate amino acids and bases as required to a final concentration of 40  $\mu$ g of each ml<sup>-1</sup>. Media were solidified with 2% Bacto agar. Cells were grown on YP agar supplemented with 2% glucose (YPD) or 2% galactose or on low-phosphate medium as previously described (33). The antibiotic G418 was added to a final concentration of 100  $\mu$ g ml<sup>-1</sup>. Fivefold serial dilutions from concentrated suspensions of exponentially growing cells ( $5 \times 10^6$  cell ml<sup>-1</sup>) were spotted onto

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TABLE 1. List of *K. lactis* strains

Strain	Genotype	Source or reference
JA6	MAT $\alpha$ <i>ade1-600 ade T-600 trp1-11 uraA1-1</i>	6
PM6-7A	MAT $\alpha$ <i>ade1-600 ade T-600 uraA1-1</i>	H. Fukuhara
MW270-7B	MAT $\alpha$ <i>metA1-1 leu2 uraA1-1</i>	M. Wesolowski-Louvel
PM4-4B	MAT $\alpha$ <i>ade1-600 adeT-600 uraA1-1</i>	24
JA6/151	MAT $\alpha$ <i>ade1-600 ade T-600 trp1-11 uraA1-1 dgr151-1</i>	25
yIG2	MAT $\alpha$ <i>ade1-600 ade T-600 trp1-11 uraA1-1 KIMIG1::URA3</i>	21
JA6/112	MAT $\alpha$ <i>ade1-600 ade T-600 trp1-11 uraA1-1 rag1kht2</i>	25

synthetic YPD agar plates containing either no drug, 1  $\mu$ g of tunicamycin (Sigma) ml<sup>-1</sup>, or 15  $\mu$ g of hygromycin B (Calbiochem) ml<sup>-1</sup>, and the plates were incubated at 30°C for 48 h. Batch experiments were conducted under selective conditions in YP medium supplemented with 2% galactose as described previously (45). *Escherichia coli* strain JM83 [*araD (lac-proAB) rpsL (=strA) f80 lacZ DM15*] was grown in Luria-Bertani medium (38). Ampicillin was added to a final concentration of 100  $\mu$ g ml<sup>-1</sup> for plasmid maintenance.

**Plasmids and transformations.** The plasmids used for this study were pYG107, carrying a cassette for the secreted expression of recombinant human serum albumin (HSA) driven by the native prepro signal sequence under the control of the *LAC4* promoter, as described by Fleer et al. (18), and pYG81, carrying the secretion cassette of interleukin-1 $\beta$  (IL-1 $\beta$ ) with the *K. lactis* killer toxin signal sequence under the control of the *PHO5* promoter (19). The *K. lactis*-*E. coli* shuttle vector pGM-GAM contained the glucoamylase (GAA) gene from the yeast *Axula adenivorans* (33). Plasmid DNA was prepared from *E. coli* by use of standard techniques (38). Yeast transformation was carried out by electroporation as described previously (50).

**Sequencing of mutation site in *dgr151* mutant.** The oligonucleotides used for *dgr151-1* amplification were as follows: KRag5F (5'-GAGCTAACGCAAAAGCTAAAC-3') and KRag5R (5'-TGGATTGTATGAGGGAATCA-3'). The product of the PCR was cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions and was sequenced (MWG Biotech) by using the same primers.

**Detection method for GAA.** GAA activity was determined by the measurement of starch hydrolysis. The starch-hydrolyzing activity of the culture medium was assayed by measuring the rate of decrease of *A*<sub>580</sub> as described previously (33). A few changes in the method were introduced, as follows: GAA activity was usually estimated at 37°C, samples were not cooled on ice, and one unit of GAA activity was defined as the quantity of enzyme needed to decrease the absorbance at 580 nm by one absorbance unit per minute.

**Northern blot analysis.** Total RNAs were prepared by extraction with hot acidic phenol (4). Northern blot analysis was performed as described previously (38). The *A. adenivorans* GAA probe corresponded to the 1.4-kb HindIII region derived from the pGM-GAM plasmid (33). The *KLACT1* probe corresponded to the 1.4-kb HindIII region derived from a *KLACT1*-containing pUC19 plasmid. The probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP by use of the Ready Prime DNA labeling system (Amersham) according to the manufacturer's instructions.

**Analysis of extracellular invertase.** The preparation of invertase extracts, nondenaturing polyacrylamide gel electrophoresis (PAGE) of invertase, and detection of enzyme activity by staining were done as described by Ballou (5), with the following minor modifications: the concentration of polyacrylamide was raised to 3.5% (wt/vol) and 15-cm-long slab gels were run for 18 h at a 15 mA constant current. These modifications allowed us to better resolve the highly glycosylated forms of invertase. For enzymatic deglycosylation, 500 U of endoglycosidase H (endo H; New England Biolabs) was added to the samples and incubated for 18 h at 37°C.

**Analysis of IL-1 $\beta$ .** An amount of culture supernatant corresponding to 10<sup>9</sup> cells was mixed with an equal volume of 20% trichloroacetic acid. The precipitated proteins were collected by centrifugation (15 min at 14,000  $\times$  g). The pellets were washed with acetone, air dried, mixed with 30  $\mu$ l of loading buffer (0.1 M Tris-HCl [pH 7.4], 20% glycerol, 4% sodium dodecyl sulfate [SDS], 5%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue), and examined by SDS-10% PAGE. The secreted production of IL-1 $\beta$  was evaluated by Coomassie blue R-250 staining of the media from the cultures after gel electrophoresis.

**Analysis of chitinase.** Native chitinase was purified from stationary cultures of *K. lactis* grown in YP medium with 2% glucose as a carbon source by use of a

method described by Kuranda and Robbins (28) and was detected by Western blotting using rabbit immunoglobulin G (IgG) antibodies against deglycosylated chitinase at a 1:3,000 dilution (a generous gift of W. Tanner, University of Regensburg, Regensburg, Germany). The final visualization was obtained with an anti-rabbit IgG conjugated with peroxidase and by use of an ECL detection kit (Amersham).

**Analysis of recombinant HSA.** For batch experiments, an amount of culture medium corresponding to 14  $\mu$ g of cell dry weight was directly loaded in an SDS-10% PAGE gel, and after electrophoresis, was electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) in Towbin buffer at 100 V for 1 h. The primary polyclonal antibodies were used at a 1:10,000 dilution (Sigma). The secondary antibody was an anti-rabbit IgG conjugated with peroxidase (Bio-Rad). An ECL detection kit (Amersham) was used according to the manufacturer's instructions. Densitometric analysis was performed with an image analyzer (Phoretix 1D; Non-Linear Dynamics Ltd.) and was normalized against a different standard of HSA (Sigma).

## RESULTS

**GAA production is strain dependent.** The yeast *A. adenivorans* secretes high amounts of GAA into culture media. The *GAA* gene encoding this enzyme has been cloned, and the cloned gene was fused to the strong *GAPDH* promoter of *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase for use as a tool to study protein secretion (8, 31, 36). This construct, introduced in a *K. lactis* multicopy vector, was named pGM-GAM (33) and was used for this study as a reporter. The cloned *GAA* gene retains the coding information for the 16 N-terminal amino acid residues and this native signal peptide is correctly cleaved during transport to the *K. lactis* cell surface (8).

The relevance of the host genetic background to heterologous protein production is well known; we therefore used four *K. lactis* laboratory strains, namely JA6, PM4-4B, PM6-7A, and MW270-7B, that were transformed with the plasmid pGM-GAM. The secretion efficiencies of the transformants, grown on 2% glucose, were compared by measuring the activity of GAA released into the culture medium and were normalized by cell number (Fig. 1A). Strain JA6 was found to be the best secretor strain.

On the other hand, the JA6 strain is known to be one of the most sensitive *K. lactis* strains for glucose repression (25, 47), a drawback for biomass-dependent industrial applications. We next analyzed the GAA production by JA6 grown on different glucose concentrations. The production of GAA was affected by the glucose concentration in the medium: the activity released into the culture medium by JA6 grown on 5% glucose was only one-third that obtained on 0.2% glucose (Fig. 1B).

We then asked whether JA6 mutants that were relieved from glucose repression would have further improvements of the secretory capabilities found in the parent strain.

**Analysis of GAA production in non-glucose-repressible mutants *dgr151-1*, *Klmig1 $\Delta$* , and *rag1 kht2*.** We analyzed whether mutations in the *RAG1*, *DGR151*, and *KLMIG1* genes, when present in the JA6 background, could also affect GAA production, since these genes are involved in glucose repression. The *RAG1* locus is polymorphic within *K. lactis* species; in strain JA6, this locus encompasses *RAG1* and a second gene, *KHT2*, which is highly similar and contiguous to *RAG1* (7). The double mutant *rag1 kht2* was thus analyzed in the JA6 background. The mutants were transformed with the pGM-GAM plasmid and grown on 2% glucose as a carbon source in the presence of G418 (Geneticin).

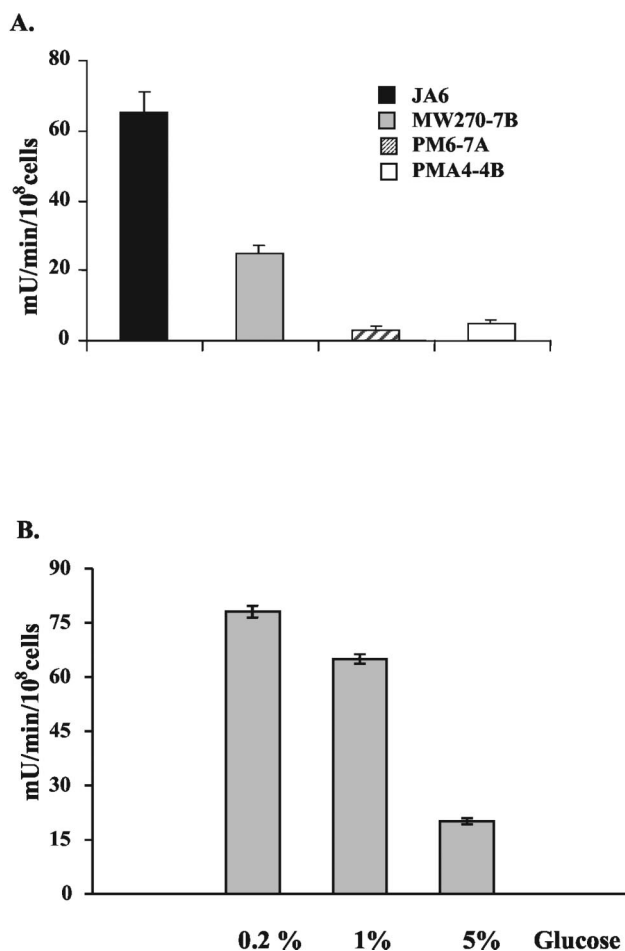


FIG. 1. Secretion of *A. adenivorans* GAA from four laboratory strain of *K. lactis*. (A) Four typical laboratory strains (PM4-4B, JA6, PM6-7A, and MW270/7B [see Table 1]) were transformed with the plasmid pGM-GAM carrying the *GAA* gene of *A. adenivorans*. The transformed strains were grown to late exponential phase in liquid YP medium containing 2% glucose and monitored by the counting of cells with a Burkholder chamber, and the GAA activity in the supernatants of cultures was determined and normalized by cell number (see Materials and Methods). The reported values are the means of three independent experiments. (B) Strain JA6 transformed with plasmid pGM-GAM was grown to late exponential phase in liquid YP medium containing the indicated amount of glucose and monitored by the counting of cells with a Burkholder chamber; the levels of amylase production in the culture medium was then determined as for panel A. The reported values are the means of at least three independent experiments.

We analyzed the efficiency of secretion by comparing the amount of GAA activity released into the growth medium normalized by cell number (Fig. 2A). The maximum amount of GAA was obtained when *dgr151-1* cells were used as the host, whereas the *Klmig1Δ* and *rag1 kht2* mutants secreted smaller amounts of GAA than did their parental counterparts. The *dgr151-1* mutant remained the best producer of GAA in the medium when cells were also grown on 2% lactose or galactose (not shown).

We then analyzed the levels of *GAA* mRNA from *dgr151-1*, *Klmig1Δ*, and *rag1 kht2* cells and from the isogenic parental strain JA6 to determine whether the observed differences could be attributed to a transcriptional alteration of the *GAA*

gene. In the wild-type strain, the level of *GAA* transcript was higher than that measured in all of the mutants (Fig. 2B). It is worth noticing that, although the amount of *GAA* mRNA from *dgr151-1* cells was lower than that from wild-type cells grown under the same conditions (compare lanes 3 and 1), the secreted GAA activity from the mutant strain was much higher than that from the wild type.

***dgr151-1* as host for recombinant protein production.** The increased production of GAA suggested that alterations in protein synthesis or release may occur when *RAG5* is mutated. We therefore analyzed the secretory capabilities of the *dgr151-1* strain by utilizing two other reporter proteins, IL-1 $\beta$  and HSA. Wild-type and *dgr151-1* cells were transformed with the plasmid pYG81, which carries the IL-1 $\beta$  cDNA fused to the secretion signal of the *K. lactis* killer toxin ( $\alpha$  subunit) (19). The

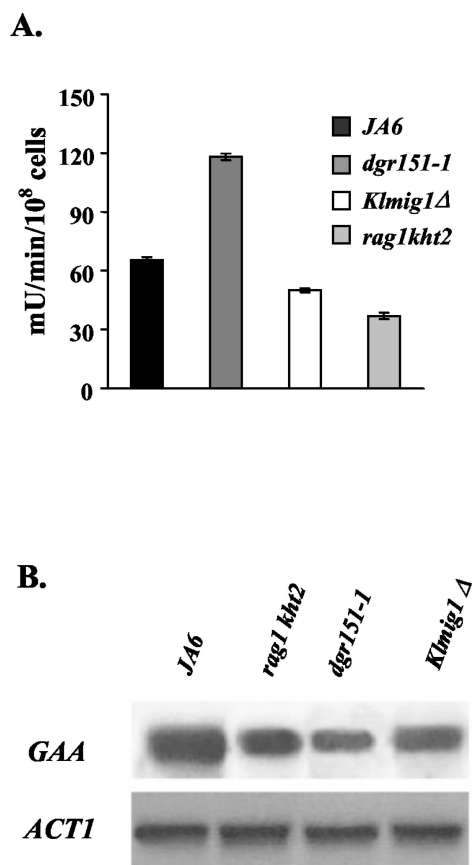


FIG. 2. GAA production by *rag1 kht2*, *Klmig1Δ*, and *dgr151-1* mutants and the isogenic parental JA6 strain. (A) Mutants JA6/112 (*rag1 kht2*), yIG2/1 (*Klmig1Δ*), and JA6/151 (*dgr151-1*) and parental JA6 cells, upon transformation with the pGM-GAM vector, were grown in liquid YP medium containing 2% glucose to late exponential phase and monitored by the counting of cells with a Burkholder chamber; the GAA activity released into the culture medium was then determined and normalized by cell number. The reported values are the means of at least three independent experiments. (B) Northern blot analysis of *GAA* expression in *rag1 kht2*, *Klmig1Δ*, and *dgr151-1* mutants and their isogenic parental strain. The strains were grown in liquid YP medium containing 2% glucose to late exponential phase, and total RNAs were extracted. After electrophoresis and blotting, the RNAs were hybridized to a labeled *GAA* probe (top). As a loading control, a *KLACT1* probe was included (bottom).

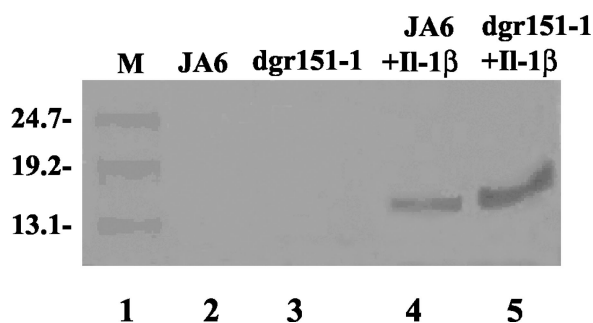


FIG. 3. Secretion of IL-1 $\beta$  from *dgr151-1* and wild-type cells. Medium aliquots corresponding to  $10^9$  cells of strain JA6 and the *dgr151-1* mutant transformed with pYG81 (lanes 4 and 5) or with an empty plasmid (lanes 2 and 3) were loaded in each lane. IL-1 $\beta$  was detected by gel staining with Coomassie blue R-250. M, molecular mass markers.

amount of IL-1 $\beta$  in the culture medium was analyzed by SDS-PAGE after 3 days of growth on YPD medium deprived of phosphate to induce transcription controlled by *PHO5*. Each lane was loaded with an amount of medium corresponding to  $10^9$  cells (Fig. 3). Densitometric measurements of the IL-1 $\beta$  bands indicated that the amount of IL-1 $\beta$  secreted from *dgr151-1* cells was nearly twice that released from the wild-type cells. We then performed a batch production experiment with wild-type and mutant cells transformed with plasmid pYG107, which carries the HSA cDNA, including the native prepro signal sequence that is known to be correctly processed in *K. lactis* (18). The cultures were maintained for 11 days, and the HSA secreted into the culture medium was detected by Western blot analysis. The data, normalized by cell mass and by a comparison with known amounts of commercial HSA used as a standard, are graphically reported in Fig. 4. Again, the *dgr151-1* cells secreted about two times more HSA than did the parental cells over the entire batch extension. The cell mass yields were highly similar for the mutant and wild-type strains. Since we used three different reporter proteins, or three different secretion signals, the enhanced secretory capabilities of *dgr151-1* mutant cells do not seem to be related to a particular protein.

In order to clarify the nature of the *dgr151-1* mutation, we determined the nucleotide change in the *dgr151-1* allele (Fig. 5). The products of two independent PCR amplifications of the gene were directly sequenced, and the *dgr151-1* allele was also cloned from a third independent amplification in the pCRII vector (pCRII-*dgr151*) and then sequenced. The obtained sequences were compared with the wild-type *RAG5* coding sequence (EMBL accession number X61680). For all three amplifications, the same single nucleotide change was found, namely a G $\rightarrow$ A transition at nucleotide 527 causing a change from glycine 176 to aspartic acid (G176D). This change is located in the highly conserved kinase domain, and it is worthwhile to remember that this mutant only retains about 5% of the parental hexokinase activity (25).

**Altered glycosylation in *dgr151-1* cells.** Hexokinase II, which converts hexoses to hexose phosphates, plays a central role in carbohydrate metabolism; a strong reduction in its activity may affect the supply of D-mannose derivatives, which are needed for glycosylation reactions as well as cell wall morphogenesis (9). We therefore looked for cell wall- and glycosylation-related phenotypes of the *dgr151-1* mutant. We first analyzed the

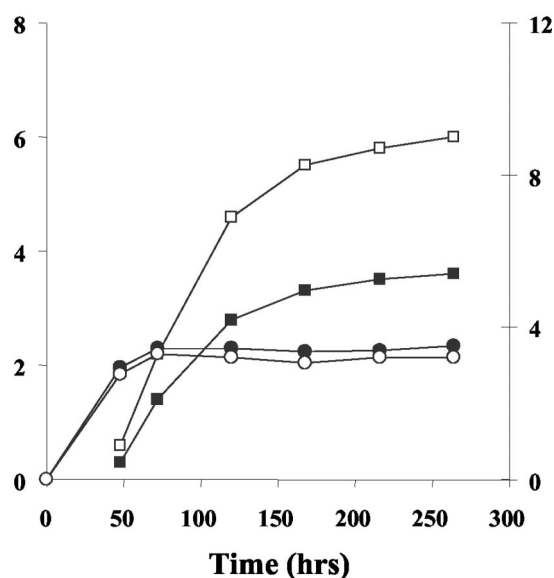


FIG. 4. Secretion of HSA from *dgr151-1* and wild-type cells. The *dgr151-1* mutant (strain JA6/151; open symbols) and the wild-type strain (JA6; closed symbols) were transformed with the plasmid pYG107 carrying the HSA cDNA and were grown in shake flasks in batch cultures in YP medium containing 2% galactose. The production of biomass (grams per liter of dry mass; right y axis) (circles) and secretion of HSA into the culture medium (milligrams per gram of cell dry mass; left y axis) (squares) were determined over the course of 11 days. Culture samples were removed at intervals and analyzed by SDS-PAGE; each lane was loaded with an amount of culture medium corresponding to the same cell number ( $5 \times 10^6$ ). The SDS-PAGE bands were transferred to a polyvinylidene difluoride membrane and then immunoreacted with recombinant HSA antibodies. The values reported are the means of three determinations with <3% variation.

tunicamycin sensitivity of the mutant; this drug is an inhibitor of the initial step of biosynthesis of the core oligosaccharide in the N-linked glycosylation pathway (41). *dgr151-1* cells were found to be hypersensitive to this inhibitor compared to their wild-type counterpart (Fig. 6). Since the glycosylation mutants of *S. cerevisiae* have often been reported to be hypersensitive to aminoglycosides (12), we also tested the resistance of the mutant cells to hygromycin B. We indeed found that *dgr151-1* cells were hypersensitive to this drug compared to the wild type (Fig. 6). In order to investigate the glycosylation defects of this mutant, we used invertase as a reporter protein that is glycosylated along the secretory pathway. The invertase extracted from the *K. lactis* wild-type strain appeared, upon native gel electrophoresis, as a diffuse band in the upper part of the gel due to the heterogeneous N-linked glycosylation of the protein (Fig. 7A, lane 1). For the *dgr151-1* strain, the diffuse band showed a faster mobility, suggesting a partial defect of N-linked glycosylation (Fig. 7A, lane 2). This interpretation was confirmed by treating the protein extracts with endo H; no difference in the migration of invertase produced by the two strains was detected (Fig. 7A, lanes 3 and 4).

We then analyzed the O-linked glycosylation of the *dgr151-1* strain, employing as chitinase as a reporter. This protein is secreted from yeast cells into the growth medium and is exclusively O-mannosylated (28). Chitinases from the mutant and the wild type were purified from the culture medium by selec-

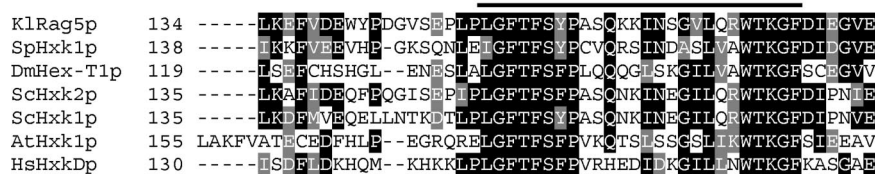


FIG. 5. Multiple alignment of proteins related to Rag5p. The sequences used for the comparison were from the following organisms: *K. lactis* (Rag5p), *Schizosaccharomyces pombe* (SpHxk1p), *Drosophila melanogaster* (DmHex-T1p), *S. cerevisiae* (ScHxk2p and ScHxk1p), *Arabidopsis thaliana* (AtHxk1p), and *Homo sapiens* (HsHxkDp). The bar indicates the characteristic kinase domain of hexokinases. The arrowhead marks the site of mutation of *dgr151-1*. The mutation corresponds to a replacement of the invariant G with D at position 176.

tive chitin binding. The subsequent analysis by SDS-PAGE was based on the notion that the electrophoretic mobility of chitinase depends on the amount of O-linked mannose (28). The data obtained showed that the chitinase secreted by mutant cells had a faster mobility than that produced by their wild-type counterparts, suggesting that the amount of O-linked mannose is also reduced in the *dgr151-1* strain (Fig. 7B).

Defects in glycosylation processes are expected to affect the assembly of cell walls by yeasts. We tested the cell wall integrity by analyzing the sensitivity of the *dgr151-1* mutant to cell lysis induced by a treatment with Zymolyase, a commercial preparation of  $\beta$ -1,3-glucanase. Mutant cells were sensitive to enzymatic lysis to the same extent as their wild-type counterparts, indicating that no significant cell wall alterations occurred in *dgr151-1* cells (data not shown).

## DISCUSSION

The yeast *K. lactis* is one of the model systems utilized for heterologous protein production in the food and pharmacology industries (18, 19, 35, 39, 44, 51). In this work, we reported the characterization of a non-glucose-repressible mutant of *K. lactis* to be used as a host for recombinant protein production.

The *A. adenivorans* GAA was chosen as a tool to study protein secretion. Strain JA6 was the best producer among the wild-type strains analyzed and was chosen for detailed analyses. This strain has been known to be particularly glucose sensitive, which is generally regarded as a negative trait for industrial applications. We therefore examined the external release of heterologous proteins by the available glucose repression-defective mutants of this strain. Among them, only the *dgr151-1* mutant produced a higher amount of GAA under all conditions analyzed than did the parental JA6 strain. This

result may suggest that the release from glucose repression per se is not sufficient to improve the secretory performance of *K. lactis* strains; on the other hand, an unexpected link between the secretory process and the activity of hexokinase can be pointed out. The increased secretory capability was not restricted to GAA secretion, as it was in fact also observed for human IL-1 $\beta$  and HSA under different secretion signals. The mutation in *dgr151-1* cells resides in the *RAG5* gene coding for the single hexokinase of *K. lactis* (25, 34). This mutation resulted in an amino acid change within the kinase domain of the enzyme, thus explaining the severely reduced kinase activity reported for this mutant (25). The enhanced secretory capability of the *dgr151-1* mutant is not related to cell wall alterations since this mutant was not affected in Zymolyase sensitivity and did not show any lysis defects (our unpublished results).

It has been known that mutations in *DGR151/RAG5* affect glucose repression in *K. lactis* (25), and in *S. cerevisiae*, a mutation of hexokinase II led to a deficiency of glucose repression (16). Indeed a *rag5* mutation in *K. lactis* can be complemented by the hexokinase II gene of *S. cerevisiae* (34).

Recent reports have demonstrated that, for *S. cerevisiae*, mutants with altered relative levels of glucose-6-phosphate (Glc-6-P) and glucose-1-phosphate (Glc-1-P) (2) or that are unable to convert glucose to Glc-6-P (32) show alterations in cellular  $\text{Ca}^{2+}$  homeostasis. Also, in the case of our study, a strongly reduced hexokinase activity could conceivably diminish the availability of Glc-6-P, thus altering the relative balance with Glc-1-P in the cell. Calcium signaling plays a crucial role in many cell processes; the relevance of  $\text{Ca}^{2+}$  to the functioning of the secretory machinery is a feature that is conserved from yeasts to human cells (10, 29). Alterations in cell calcium homeostasis have been observed in an *S. cerevisiae* *PMR1*-disrupted mutant; *PMR1* encodes a  $\text{Ca}^{2+}$ -ATPase that is localized in the Golgi apparatus (3, 15). The inactivation of this gene also resulted in a significant increase in the secretion of several heterologous proteins (26, 37). We could thus speculate that the oversecretion phenotype of the *dgr151-1* mutant could also be linked to alterations in cell  $\text{Ca}^{2+}$  homeostasis that, in turn, originate from a perturbed balance of Glc-1-P and Glc-6-P.

The availability of the *dgr151-1* mutant is relevant for biomass-directed industrial applications, since in contrast with the *rag5* $\Delta$  mutant (34), *dgr151-1* cells are able to utilize different carbon sources, such as glucose and lactose. This ability could be due to the residual hexokinase activity, which might dimin-

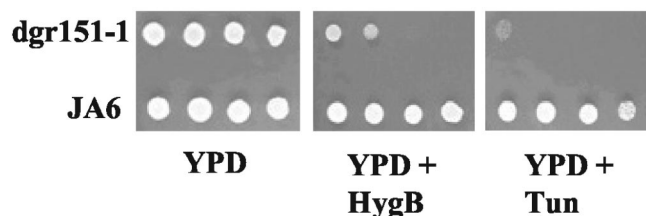


FIG. 6. *dgr151-1* mutant is hypersensitive to tunicamycin and hygromycin B. *dgr151-1* and wild-type (JA6) cells were grown in YPD medium. Serial dilutions of cultures were spotted onto YPD plates in the presence of tunicamycin (Tun) (1  $\mu\text{g ml}^{-1}$ ) or hygromycin B (HygB) (15  $\mu\text{g ml}^{-1}$ ) as indicated.

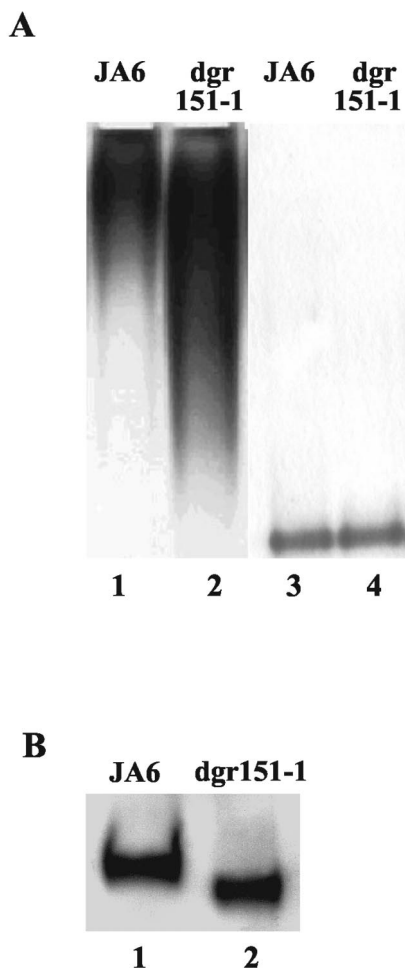


FIG. 7. Reduced glycosylation in *dgr151-1* mutant. (A) Effect of *dgr151-1* mutation on native invertase glycosylation. Cells were grown in YPD medium and total cell proteins corresponding to  $10^8$  cells were subjected to native gel electrophoresis (3.5% polyacrylamide) and stained for invertase activity as described in Materials and Methods. Fully glycosylated invertase was present in lane 1 (wild-type strain JA6), and underglycosylated invertase was produced by *dgr151-1* cells (lane 2). Portions of the samples were treated with endo H (lanes 3 and 4). (B) Immunoblot analysis of chitinase secreted from *dgr151-1* and JA6 cells. O-glycosylated chitinase was isolated from wild-type (lane 1) and *dgr151-1* (lane 2) cells as described in Materials and Methods, subjected to SDS-PAGE, and then immunoreacted with chitinase antibodies.

ish the intracellular accumulation of unphosphorylated glucose that is regarded as deleterious for the cells (35).

The reduced mannose extension of N- and O-glycoproteins observed for *dgr151-1* cells could be ascribed to a reduced availability of GDP-mannose, the substrate for the glycosylation reactions that occur in the Golgi apparatus (46). The substrate for glycosyltransferases is in fact synthesized in the cytosol, starting from mannose-6-phosphate (Man-6-P), which is obtained by transforming fructose-6-phosphate, which in turn originates from the Glc-6-P produced by the hexokinase (9). The *S. cerevisiae* phosphomannomutase, the product of the *SEC53* gene, converts Man-6-P to mannose-1-phosphate (Man-1-P), the direct substrate for the formation of GDP-mannose (27). In *S. cerevisiae*, three enzymes, namely Hxk1p,

Hxk2p, and Glk1p, are able to phosphorylate the hexoses at C6, while in *K. lactis*, Rag5p is regarded as the unique hexokinase (34). The *dgr151-1* mutation, by strongly impairing the hexokinase activity (25), would result in a reduced supply of Man-1-P that is not sufficient for a wild-type level of glycosylation. The reduced glycosylation level, however, seems not to have a direct relationship with the increase in secretory capabilities we observed for *dgr151-1* cells, since the HSA and IL-1 $\beta$  that we used as reporters are not glycosylated proteins and their transport, therefore, should not be affected by the glycosylation process.

Taken together, our data point out the *dgr151-1* mutant strain as a useful host for heterologous protein production, as it provides the possibility not only for obtaining a higher amount of recombinant proteins but also for avoiding the hyperglycosylation of secreted proteins.

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